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2013; mAbs 5(5)
http://dx.doi.org/10.4161/mabs.25743

http://www.landesbioscience.com/journals/mabs/article/25743/

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A unique anti-CD115 monoclonal antibody which inhibits osteolysis and skews human monocyte differentiation from M2-polarized macrophages towards dendritic cells

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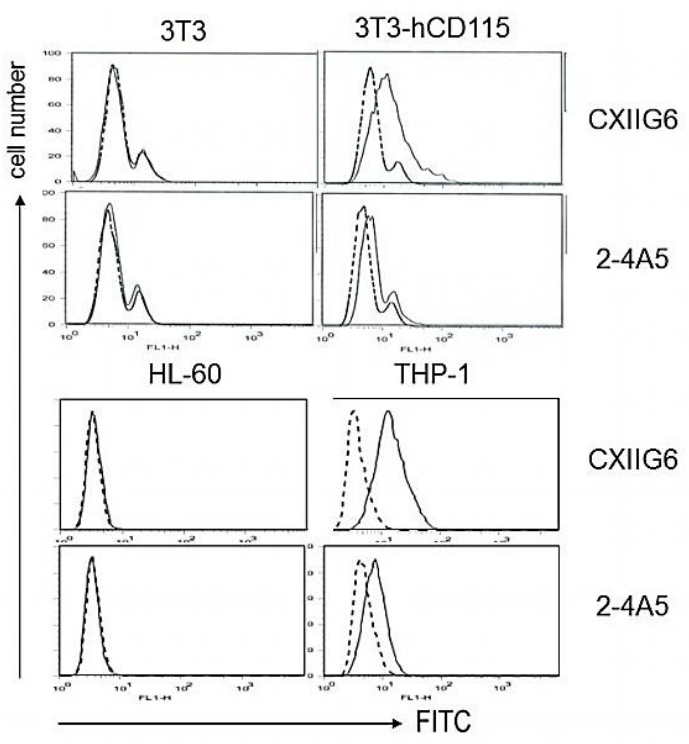
Supplementary Figure 1. Murine IgG_{2a} CXIIG6 binds to human CD115

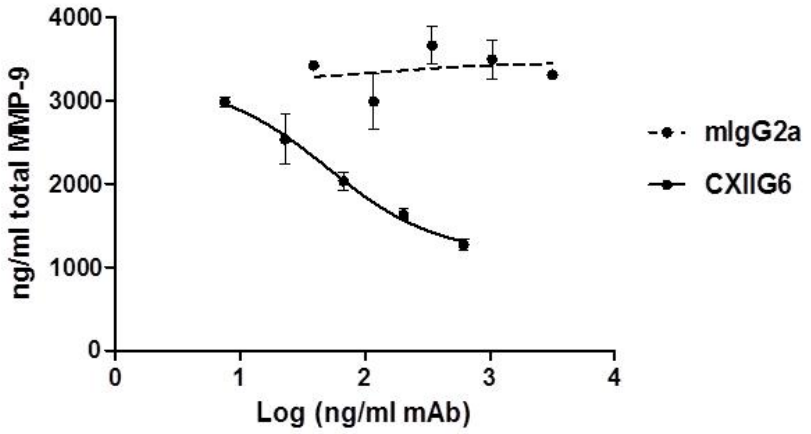
Hybridomas were generated from immunized mouse splenocytes and clone CXIIG6 was selected as described in the Supplementary Methods. Anti-CD115 mAb binding to cell lines NIH/3T3, NIH/3T3-CD115, HL-60 and THP-1 was analyzed by FC. Immunostaining with CXIIG6 hybridoma supernatant (followed by goat anti-mouse $IgG+M^{FITC}$) was compared with that obtained using the commercial anti-CD115 mAb 2-4A5[1] (followed by mouse anti-rat IgG^{FITC}) (plain-lined histograms). Dotted lines correspond to isotype controls.



Culture supernatant from hybridoma CXIIG6 stained CD115-transfected NIH/3T3 cell line, but not untransfected cells. It also stained the human monocytic leukemia cell line THP-1, but not the promyelocytic leukemia cell line HL-60, which was surface CD115-negative.

Supplementary Figure 2. **Murine mAb CXIIG6 inhibits secretion of MMP-9 by monocyte-derived osteoclasts cultured with CSF-1 and RANKL**

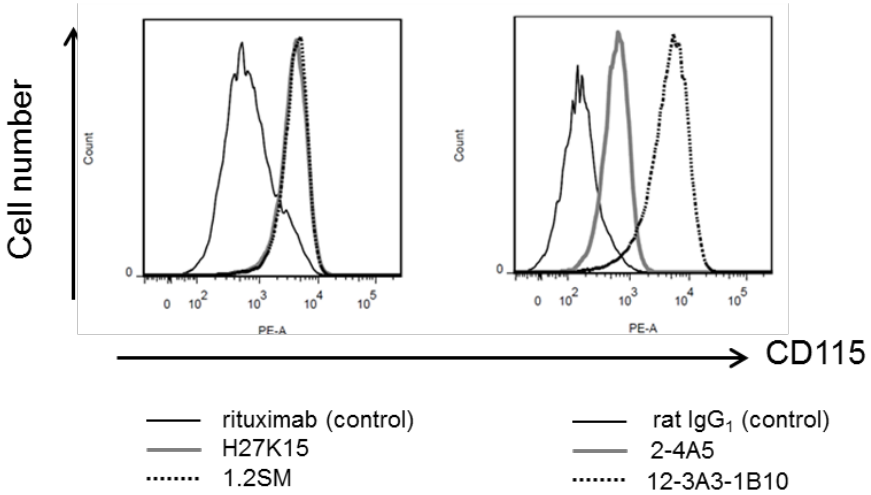
Primary human monocytes cultured in the presence of human CSF-1 and RANKL differentiate into osteoclasts, which secrete MMP-9[2]. We used this model to investigate whether recombinant murine mAb CXIIG6 might affect monocyte differentiation towards osteoclasts. Monocytes obtained by elutriation of peripheral blood cells from a healthy donor were seeded at 2 x 10^4 cell/well in 96-well plates, in complete alpha-MEM medium supplemented with 10 % FCS. They were treated for 45 min with serial dilutions of mAb CXIIG6 or isotype control IgG_{2a} (R&D Systems). Complete culture medium containing hCSF-1 (25 ng/ml, Immunotools) and RANKL (40 ng/ml, PeproTech,) was then added in a final volume of 200 µl/well. Every 2 to 3 days, 150 µl culture medium were replenished with cytokines and mAbs until day 8. Culture supernatants conditioned for 72 h were titrated for human MMP-9 by ELISA (R&D Systems). Means +/- SEM of triplicate wells are shown.



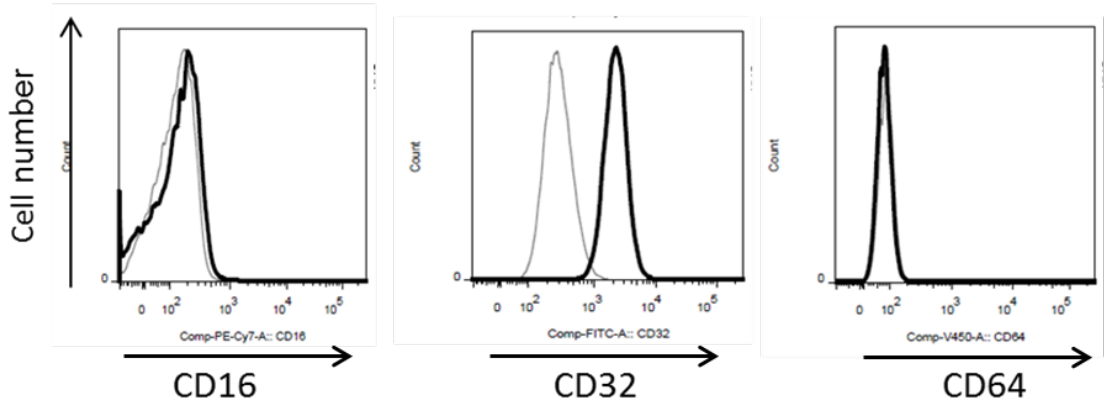
When the anti-CD115 mAb was added to the cultures, levels of MMP-9 in day-8 supernatants were found to decrease dose-dependently. This suggested that mAb CXIIG6 could inhibit the differentiation of osteoclasts by affecting CD115 receptor function.

Supplementary Figure 3. Surface expression of CD115 and FcγR by OCI-AML5 cells

A. After overnight culture in the absence of CSF-1, OCI-AML5 cells were immunostained with the anti-CD115 mAbs H27K15, 1.2SM or isotype control rituximab (left panel), or with 2-4A5, 12-3A3-1B10 or isotype control rat IgG_1 (right panel).

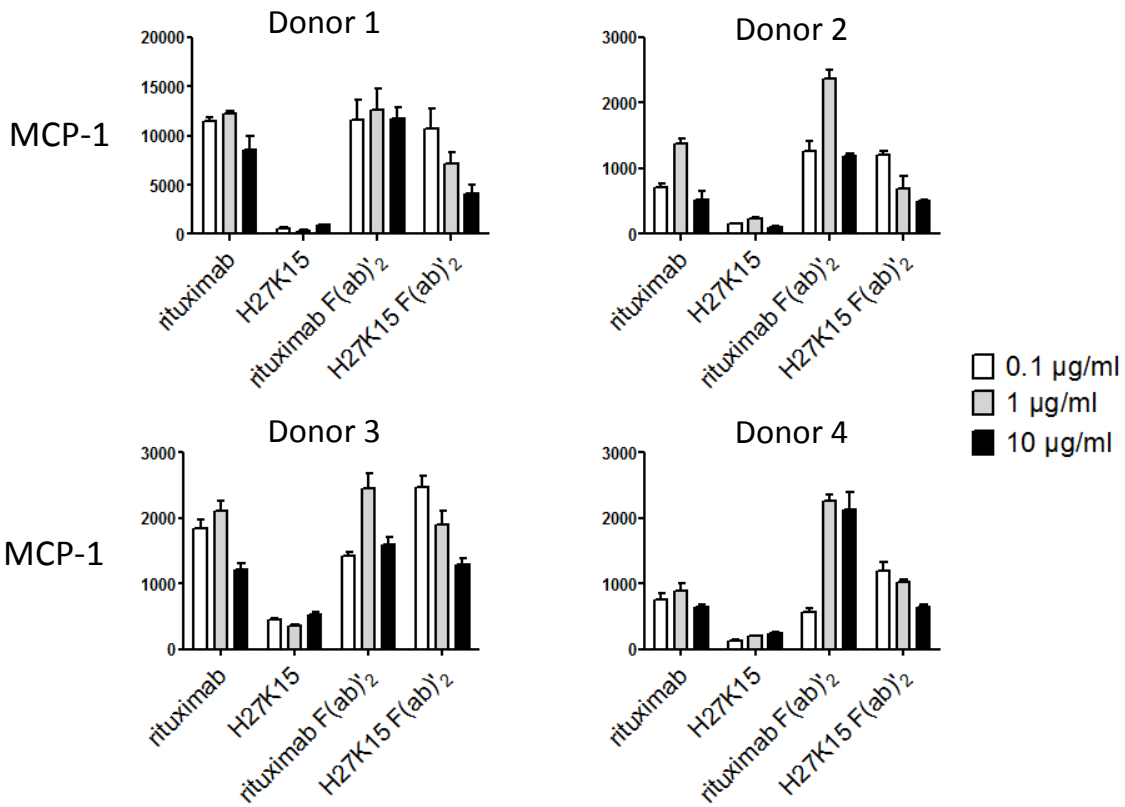


B. OCI-AML5 cells were immunostained with fluorochrome-conjugated murine $IgG_{1,\kappa}$ anti-CD16 (clone 3G8), anti-CD32 (clone 3D3, recognizing both FcγRIIa and FcγRIIb) and anti-CD64 (clone 10.1) (black line histograms). Isotype controls are shown in grey. OCI-AML5 cells stain negatively for CD16 and CD64, but express high surface levels of CD32.



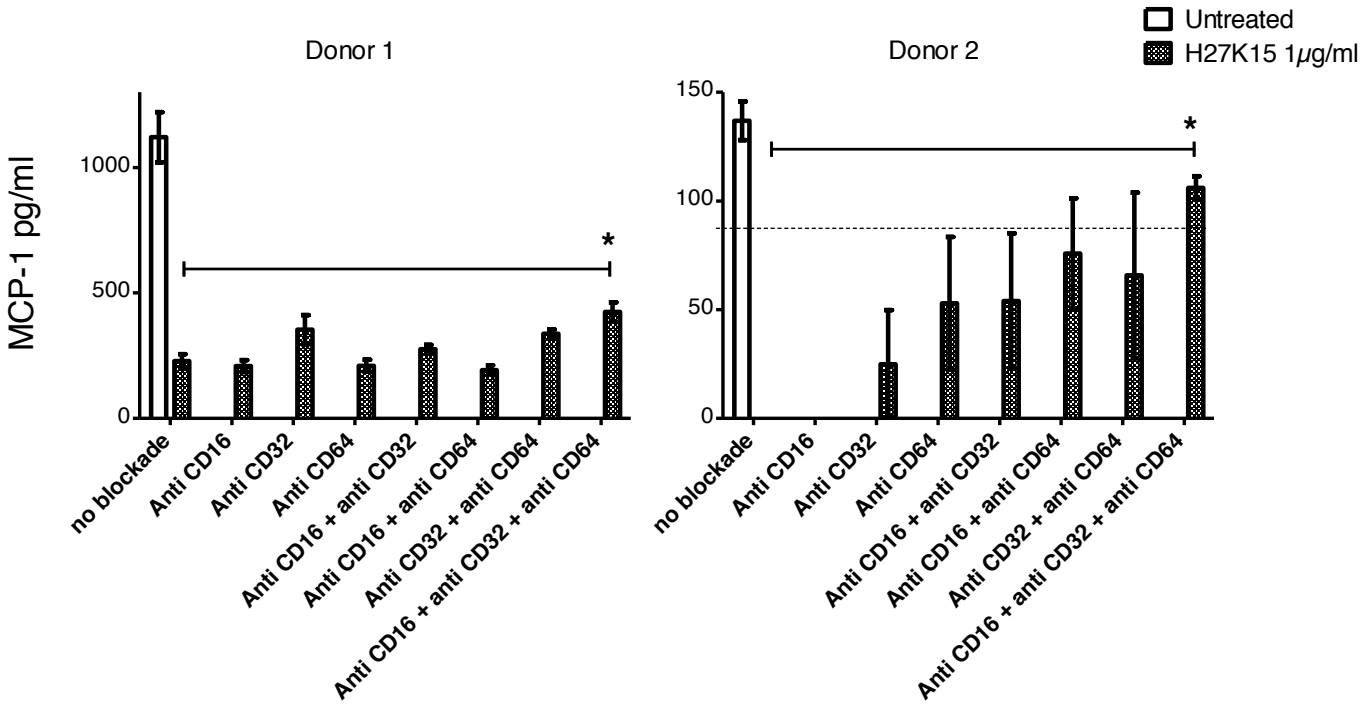
Supplementary Figure 4. MCP-1 secretion in monocyte-to-macrophage differentiation cultures treated with anti-CD115 mAb H27K15 or derived F(ab)'$_2$

MCP-1/CCL2 was titrated by FlowCytomix (eBiosciences) in culture supernatants from monocyte-derived cells from 4 different blood donors after a 6-day culture with GM-CSF and CSF-1 in the presence of mAb H27K15 or isotype control Rituximab at the indicated concentrations, or their derived F(ab)'$_2$ at corresponding molar concentrations. Results shown are means +/- SD from triplicates.



Supplementary Figure 5. Contribution of FcγR in the inhibition of MCP-1 production by mAb H27K15

MCP-1/CCL2 was titrated by ELISA (R&D Systems) in culture supernatants from monocyte-derived cells from 2 different blood donors after a 6-day culture with GM-CSF and CSF-1 in the presence of 1 µg/ml of mAb H27K15, with or without blocking F(ab')2 to CD16, CD32 and CD64 (Ancell, 6 µg/ml added on days 0 and 3) alone or in combinations. Limit of MCP-1 detection was 90 pg/ml (hatched line on the right graph). Results shown are means +/- SEM from quadruplicate wells. * Mann-Whitney test $p<0.05$ vs H27K15 treatment without FcγR blockade.



H27K15-mediated inhibition of MCP-1 secretion was affected by CD32 blockade in cultures from Donor 1, and by either CD32 or CD64 blockade in cultures from Donor 2. For both donors, only the combination of the 3 $F(ab')_2$ anti-CD16, -CD32 and -CD64 significantly inhibited the effect of mAb H27K15, suggesting that several FcγR may be involved in the mAb mode of action.

Supplementary Methods

Mouse immunization and hybridoma generation. Seven-week-old BALB/c mice (Charles River Laboratories France) were immunized by intramuscular injections of a plasmid driving the expression of human CD115 extracellular domain fused with the tetanus toxin P30 T helper epitope[3]. A boost was performed 3 days before hybridoma derivation by subcutaneous injection of 10 µg soluble human CD115-Fc (R&D Systems). Splenocytes from an immunized mouse were fused with P3X63Ag8 myeloma cells (ECACC cat. #85011401). Hybridomas were selected by culture in semi-solid ClonaCell^{TM}-HY Medium D (StemCell Technologies) for 20 days. Clones were picked and grown individually in ClonaCell^{TM}-HY Medium E (StemCell Technologies). Hybridoma CXIIG6 was subcloned and further adapted for growth in RPMI-1640 medium containing 10 % FCS, 2 mM glutamine, 40 µg/ml gentamycin, 1 mM sodium pyruvate and 0.1 mM non-essential amino acids.

Hybridomas were screened both by ELISA on recombinant human CD115 and by immunocytochemistry and FC on THP-1 cells (CD115-positive) or HL-60 cells (surface CD115-negative) (ATCC). For the detection of anti-CD115 antibodies by ELISA, 96-well microtiter plates (Maxisorp, Nunc) were coated with 100 ng human CD115-Fc (R&D Systems) per well. Following washing and saturation of the plates, hybridoma culture supernatants or mAbs were added for 1 h at 37°C. Bound antibodies were detected with horseradish peroxidase (HRP)-conjugated goat anti- mouse polyvalent Ig or goat anti-mouse IgG Fc (Sigma) followed by a 3,3',5,5'-tetramethylbenzidine (Sigma) substrate solution. Absorbance was measured at 450 nm. FC was performed as described below (Immunocytochemistry and FC).

Generation of humanized mAb H27K15. Light and heavy IgG chains were cloned from hybridoma CXIIG6 and sequenced (SEQ ID NO:1 and NO:3, patent application WO2009/112245). A chimeric IgG_1 form was generated by fusion of the sequences encoding murine variable regions (SEQ ID NO:6 and SEQ ID NO:9, WO2009/112245) with human IgG_1 constant region (GeneBank accession number J00241) or human Ig_κ constant region (GeneBank accession number: J00228). The chimeric mAb was further humanized by substitutions of murine residues in the VH and VL of chimeric CXIIG6 with human ones, selected from the closest human germline sequences. Humanized variant H27K15 (patent application WO2009/112245) was selected on the basis of its high affinity to recombinant human CD115 measured by quartz crystal microbalance (Attana), high humanization degree (85 % and 86,4 % homology with human germline IGHV3-72*1 and IGKV1-NL1*01) and lowest *in silico* immunogenicity as determined by Algonomics (BE) using Epibase®.

Antibody production. The heavy and light chains of murine (CXIIG6) or humanized (H27K15) anti-CD115 mAbs, mAb 1.2SM (IgG_1) and Rituximab were cloned respectively in plasmids pcDNA™3.3 and pOptiVEC™ (OptiCHO™ Antibody Express Kit, Invitrogen). They were expressed either by transient transfection of adherent CHO-K1 (ATCC) or CHO-DG44 cells[4] or by polyclonal pools of stable CHO-DG44 transfectants as described in the OptiCHO Antibody Express Kit. MAbs H27K15, 1.2SM and Rituximab were purified from the culture supernatants on Protein A sepharose 4FF (GE Healthcare) followed by gel filtration (HiLoad 26/60 Superdex 200 26/60, GE Healthcare). MAb concentrations in PBS solutions were calculated using the absorbance at 280 nm measured on a spectrophotometer (Analytikjena specord) and their mass extinction coefficients, determined using the Protparam software. In some instances, mAb concentrations were determined by surface plasmon resonance (SPR) using a Biacore T100[5]. Purified mAbs were sterilized by filtration on a 0.2 µm membrane (Nalgene), aliquoted and stored at -80°C. Absence of endotoxin contamination was verified using a L.A.L. test (Endosafe™ PTS, Charles River). Rituximab was also kindly provided by Roche. $F(ab')_2$ from H27K15 or Rituximab were prepared using Pierce® $F(ab')_2$ preparation kit. After digestion by pepsin, $F(ab')_2$ were purified by gel filtration (HiLoad Superdex 200 26/60, GE Healthcare) and their purity verified by Coomassie blue staining of an SDS-PAGE gel and ELISA titration of human IgG Fc.

Competition experiments by ELISA. Recombinant human CSF-1 (E33 to R445, swissprot P09603, Geneart) carrying the mutation R252A was biotinyled with biotin-NHS (Sigma) with a molar ratio of 1 Biotin-NHS/hCSF1. Ninety six-wells plates (Maxisorp, Nunc) were coated with 100 µl/well of 1 µg/ml of recombinant human CD115-Fc (R&D Systems). Serial three-fold dilutions of test protein (starting concentration: 100 µg/ml for H27K15, Rituximab and 2-4A5, 10 µg/ml for CSF-1 and 5 µg/ml for 1.2SM) were incubated with 0.06 µg/ml of CSF-1-biotin (corresponding to the concentration giving ~50 % of the maximum signal) for 1 hour at room temperature. After washing, streptavidine-HRP (Southern biotech) was added and incubated for 1 hour at room temperature. After washing, 100 µL of 3,3',5,5'-Tetramethylbenzidine (TMB) Liquid Substrate (Sigma) was added for 30 min. Optical density (OD) was recorded at 620 nm using a plate reader (Tecan).

Affinity measurements by QCM. Attana 200 (Attana) using the Quartz Crystal Microbalance (QCM) technology was used to measure the affinities of anti-CD115 mAbs for human CD115 D1-D5 fused to a poly-His tag (Geneart). Monoclonal antibodies were immobilized on LNB chip (Attana) using amine coupling kit (Attana) to reach 90 Hz, 65 Hz and 105 Hz for H27K15, 1.2SM and 2-4A5 respectively. All analyses were carried out at a flow rate of 30 µL/min and at a temperature of 37°C in HBST buffer (Attana). The sensor chip surface was regenerated after each cycle by injection of 10 mM Glycine-HCl pH3.0. CD115 D1-D5 (molecular weight 57,6 kDa) was injected at 69.4 nM, 34.7 nM, 17.4 nM and 8.7 nM for H27K15 or 8.7 nM, 4.3 nM, 2.1 nM and 1.1 nM for 1.2SM and 2-4A5. CD115 D1-D5 was

injected for 70 sec, before a post-injection phase of 180 sec. Buffer was used as a reference and corresponding values subtracted for each hCD115 concentrations. Data were analyzed using Evaluation Software (Attana) and a simple 1:1 model for data fitting.

Immunocytochemistry and FC. For CD115 staining of AML5 or EL4-CD115 cells, 2 to 5 x 10^5 cells in 200 µl PBS were incubated with 10 µg/ml of primary mAb: anti-CD115 mAbs H27K15, 1.2SM (human IgG_1) or 2-4A5 (rat IgG_1, Santa Cruz), or isotype controls Rituximab (chimeric IgG_1) or rat IgG_1 (R&D Systems). MAb binding was detected using R-PE goat anti-human Kappa light chain (Southern Biotech) or R-PE mouse anti-rat IgG (eBioscience). In some instances, cells were fixed with Cell-Fix (BD Biosciences). Fluorescence intensity was measured on a FACSCanto™ flow cytometer (Becton Dickinson) using software CellQuest Pro (Becton Dickinson) and analyzed with FlowJo (Tree Star).

For immunostaining of monocyte-derived cultures, cells were first incubated for 20 min at 4°C in PBS containing 10 % human AB serum or 10 µg/ml IgG Fc (Rockland) to saturate Fc receptors. Fluorochrome-conjugated mAbs (anti-CD32-PE or -FITC, anti-CD64-APC or -BD Horizon, anti-CD16-PE-Cy7, anti-CD163-PE, anti-CD206, anti-CD14, anti-CD1a, BD Biosciences) were then added for 20 min at 4°C. Staining for live cells was performed with Live/Dead kit APC-H7 (BD Pharmingen). Cells were washed with PBS (5 min, 2000 rpm at 4°C) and fixed with Cell-Fix. FC analysis was performed using a FACS LSR-II (BD biosciences) with the DIVA software for acquisition and the Flow Jo software for analysis.

CD115 internalization in EL4-CD115 cells. The EL4-CD115 cell line was generated at Vectalys (France) by stable transfection of the murine lymphoma-derived T cell line EL4 (ATCC) with a lentiviral vector encoding human full-length CD115 under control of the EF1α promoter. One million EL4-CD115 cells were incubated for 20 min in 100 µl ice-cold culture medium containing 100 µg/ml of mAb in the presence or absence of 100 ng/ml CSF-1 (ImmunoTools). Temperature was then raised to 37°C for 30 min. Cells were then transferred on ice and cell-surface CD115 was detected with a primary mAb, followed by a secondary R-PE-labeled antibody. Primary mAb was 12-3A3-1B10 (eBioscience) or isotype control MAB005 (R&D Systems) and secondary Ab was R-PE donkey anti-rat IgG (eBioscience). Median fluorescence intensities were immediately measured on a FACS CANTO II flow cytometer (BD Bioscience). The percentages of CD115 loss from the cell surface were calculated as follows: 100-100*(((Test MFI– Isotype control MFI / (untreated control MFI– Isotype control MFI)) / ((Test MFI at T=0 – Isotype control MFI at T=0) / (untreated control MFI at T=0 – Isotype control MFI at T=0))).

CD115 phosphorylation assay. OCI-AML5 cells (DSMZ) were starved of growth factors for 16 h in MEMα medium containing 3 % charcoal-stripped serum and then treated with 100 ng/ml CSF-1

(Immunotools) during 3 minutes at 37°C in the presence of anti-CD115 mAbs added to the culture medium 1h prior to stimulation. H27K15, 1.2SM IgG1 or 2-4A5 or isotype controls (respectively Rituximab or rat IgG1) were used at 1µg/mL. Cells were also treated by the CD115 kinase inhibitor GW2580 (1µM LC Laboratories) or vehicle. Cells were then collected, rinsed in ice-cold PBS, lysed in buffer containing 1% Nonidet P-40, 50 mM Tris pH 8, 150 mM NaCl supplemented with protease and phosphatase inhibitors (Complete, EDTA-free and PhosSTOP, Roche Applied Science). Proteins were quantified by the Bradford assay (BIO-RAD laboratories) and Western blot was performed using antibodies anti-CD115 (C-20, Santa Cruz Biotechnology), anti-phospho-Y723 CD115 and anti-phospho-Ser473 Akt (Cell Signaling Technology) and β-Actin (Sigma), detected with HRP-labeled antibodies (DAKO) and the Immun-Star WesternC Chemiluminescent kit (BIO-RAD).

Osteoclast differentiation from human monocytes. Peripheral blood mononuclear cells from healthy donors from blood donors having given informed consent (EFS Alsace) were isolated from buffy coats (Etablissement Français du Sang, Strasbourg) by gradient centrifugation using Ficoll-Paque™ PLUS (GE Healthcare). $CD14^+$ monocytes were purified using CD14 MicroBeads (Miltenyi Biotec) and the AutoMACS Pro separator (Miltenyi Biotec). Monocytes were seeded at 2.5 or 5 x 10^4 cells/well in 96-well plates in alpha-MEM medium (Gibco) containing 10 % heat-inactivated FCS (PAA). They were treated for 45 min at 37°C with serial dilutions of mAbs or $F(ab')_2$ in complete culture medium. CSF-1 (ImmunoTools) and RANKL (PeproTech) were added at 25 and 40 ng/ml. Culture medium was replenished with mAbs or $F(ab)'_2$ and cytokines at days 2 and 4 (for a 7-day differentiation) or at day 2, 4 and 7 (for an 8-day differentiation). TRAP5b was titrated in the culture supernantants using the MicroVue™ TRAP5b EIA kit from Quidel. Three-parameter fit curves were calculated by GraphPadPrism using means +/- SEM from quadruplicate wells.

Osteoclast differentiation from human $CD34^+$ cells and activity assay. Human bone marrow-derived $CD34^+$ stem cells from 5 different donors (Lonza) were cultured on bovine bone slices (IDS Ltd) in culture medium (OCP BulletKit®, Lonza) containing 10% FCS, 8.25 ng/ml CSF-1 and 16.5 ng/ml RANKL (modified from OCP BulletKit®, Lonza)[6]. After completion of osteoclast differentiation at day 7, culture medium was replaced and cells were cultured for 3 additional days allowing them to resorb bone. Cells were treated with mAb H27K15 or rituximab at days 0, 2, 4, 7, 8 and 9. Control reagent Osteoprotegerin (OPG, Peprotech) was added at day 0 and the cysteine protease inhibitor E64 (Sigma-Aldrich) at day 7. TRAP5b and CTX were titrated in the supernatants collected at day 10 by ELISA using the BoneTRAP® and CrossLaps® kits (IDS Ltd). Six replicates were performed per culture condition. Statistical analysis was performed using one-way ANOVA followed by t-test for comparing TRAP5b concentrations between H27K15- and rituximab-treated cultures, or with Kruskall-Wallis

followed by Mann-Whitney for comparing CTX levels. The cultures were stained for tartrate-resistant acid phosphatase (TRAP) using a kit (Sigma) according to the manufacturer's instructions.

Human macrophage differentiation assay. Purified $CD14^+$ monocytes from different blood donors were cultured in 48-well plates (3 x 10^5 cells/well) in RPMI-Glutamax™ or RPMI 1640 medium supplemented with 10% FCS, containing GM-CSF (10 ng/ml, Peprotech) from day 0 to day 3 and CSF-1 (10 ng/ml, Immunotools) plus GM-CSF (2 ng/ml) from day 3 to day 6. Antibodies, $F(ab)'_2$ or the CD115 TK inhibitor GW2580 (LC Labs) were added at day 0 and day 3. At day 6, cells were counted (5 microscope fields /well), harvested and pools of triplicates were analyzed by FC as described above (Immunocytochemistry and FC). Cytokines or chemokines were titrated in day-6 culture supernatants by multiplex (Bioplex, Bio-Rad) or ELISA (R&D Systems).

Monocyte viability assay. Purified human $CD14^+$ monocytes from blood donors having given informed consent (EFS Alsace) were seeded at 4 or 5 x 10^4 cells/well in 96-well tissue culture plates (TPP) in 50 µl RPMI-1640 medium (Sigma) supplemented with 10 % heat-inactivated FCS (PAA), 2 mM glutamine and 40 µg/ml gentamycin. MAbs or $F(ab')_2$ fragments were serially diluted in the same medium and 50 µl were added to the culture wells. Plates were incubated for one day at 37°C and cell survival was assessed using the CellTiter-Glo® Luminescent Cell Viability Assay from Promega, according to the manufacturer's instructions. Cell lysates (175 µl) were transferred in white 96-well plates (Cellstar®, Greiner Bio-one) and light emission was recorded for 0.5 s in a TriStar LB 941 reader (Berthold Technologies).

1. Sherr CJ, Ashmun RA, Downing JR, et al. Inhibition of colony-stimulating factor-1 activity by monoclonal antibodies to the human CSF-1 receptor. *Blood*. 1989;73(7):1786-1793.
2. Takayanagi H. Osteoimmunology: shared mechanisms and crosstalk between the immune and bone systems. *Nat Rev Immunol*. 2007;7(4):292-304.
3. Hertz M, Mahalingam S, Dalum I, et al. Active vaccination against IL-5 bypasses immunological tolerance and ameliorates experimental asthma. *J Immunol*. 2001;167(7):3792-3799.
4. Urlaub G, Chasin LA. Isolation of Chinese hamster cell mutants deficient in dihydrofolate reductase activity. *Proc Natl Acad Sci U S A*. 1980;77(7):4216-4220.
5. Christensen LL. Theoretical analysis of protein concentration determination using biosensor technology under conditions of partial mass transport limitation. *Anal Biochem*. 1997;249(2):153-164.
6. Rissanen JP, Ylipahkala H, Fagerlund KM, Long C, Vaananen HK, Halleen JM. Improved methods for testing antiresorptive compounds in human osteoclast cultures. *J Bone Miner Metab*. 2009;27(1):105-109.